# Novel Roles for HDAC4 in Innate Immunity and Inflammasome Signaling

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**ABSTRACT**

Histone deacetylases (HDACs) are important mediators of gene expression, in which deacetylation leads to changed chromatin structure that can cause repression or activation of transcription. HDACs have also been found to modulate innate immunity through post-translational regulation; however, the mechanisms involved aren't well understood. Although pan-HDAC inhibitors have been shown to limit IL-1beta secretion in response to inflammasome activation, these broad-spectrum agents have many off-target effects. The objective of this study was two-fold: 1) to examine post-translational mechanisms that regulate cytosolic DNA sensing and inflammasome activation, two important aspects of the innate immune system, and 2) to assess the specific HDAC enzymes involved which may pave the way for more targeted therapies.

First, GFP-tagged nucleotide-binding domain (NOD)-like receptor family, pyrin domain containing 3 (NLRP3), a component of NLRP3 inflammasome, was co-transfected into HEK 293T cells along with various FLAG-tagged HDACs. NLRP3-HDAC association was then analyzed via GFP pull-down assay followed by anti-FLAG western blot, which revealed that NLRP3 associates with HDAC4. Transfection of cells with the double-stranded DNA absent in melanoma 2 (AIM2) inflammasome agonist followed by analysis by confocal microscopy revealed that HDAC4 also colocalizes with AIM2 inflammasomes. Since DNA has negative charge and HDAC inhibition increases acetylation levels, we hypothesized that HDAC4 loss of function may compromise the ability of high mobility group box (HMGB) proteins to sense cytosolic DNA. Both pharmacological inhibition of HDAC4 and knockout via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) impaired HMGB1 and HMGB2 interaction with poly dA:dT as assessed by biotin-pulldown assays and confocal microscopy. Finally, we note impaired IL-1beta secretion in HDAC4 (-/-) mouse embryonic fibroblasts relative to wild-type controls in an *in vitro* inflammasome reconstitution model. Impaired IL-1beta release in the HDAC4 (-/-) cells could be partially rescued via exogenous HDAC4 transfection, indicating that HDAC4 plays a novel,

likely post-transcriptional role in cytosolic DNA sensing and inflammasome function. Current studies are underway to further characterize the mechanism(s) at work, and to determine whether specific targeting of HDAC4 may be a possible, more targeted treatment option for autoimmune or inflammatory disorders.

**INTRODUCTION**

The immune system is responsible for maintaining homeostasis in the body. When the balance is threatened, either through the invasion of pathogens or through damage to tissues, it is activated to eliminate the threat. The immune system consists of innate immunity and adaptive immunity. Innate immunity relies on mechanisms of defense already present in the body before the infection or damage took place, and is therefore less specific than the adaptive system, but also faster in its response. When the immune system is activated it often leads to inflammation, a complex reaction in which leukocytes, or white blood cells, and plasma proteins are recruited from the blood to the tissue where they accumulate and are then activated. The goal of inflammation is to protect the body by controlling infections and promoting tissue repair. However, inflammation can often lead to tissue damage and is implicated in many diseases. Acute inflammation can damage host tissues through the proteolytic enzymes and reactive oxygen species being produced to attack the pathogens. Additionally, in cases of autoimmune diseases the immune system is activated by self-antigens and targets host cells (Abul K. Abbas, 2018).

There are many components involved in the activation of the innate immune system and inflammation, including inflammasomes. Inflammasomes are multi-complex proteins responsible for cleaving pro-IL-1β and pro-IL-18 to IL-1β and IL-18, respectively. (C. A. Yang & Chiang, 2015). There have been several inflammasomes defined to date in the literature. Inflammasomes can be described as either canonical, which are dependent on caspase-1 to process the inflammatory cytokines IL-1β and IL-18, or non-canonical, which use caspse-11 in mice, or caspase 4 and caspase 5 in humans (Pellegrini et

al., 2017). Understanding the structure of canonical inflammasomes is important in order to understand their function. Canonical inflammasomes are composed of a sensor molecule specific to the particular type of inflammasome, which then connects to caspase 1 via ASC. ASC is an adaptor protein common to all inflammasomes which consists of both a pyrin domain and a caspase activation and recruitment domain (CARD). The pyrin domain of ASC interacts with the inflammasome sensor molecules which then triggers ASC to assemble or oligomerize into a large protein speck of mostly ASC dimers. The CARD domain of ASC then serves to initiate the self-cleavage of pro-caspase 1 into caspase 1. This cleaved caspase-1 is responsible for the activation of IL-1β (Latz et al., 2013). Interleukin-1β, or IL-1β, is a pro-inflammatory cytokine that mediates acute inflammatory responses. NF-κB (nuclear factor kappa B) and AP-1 (activator protein 1) transcription factors are activated when IL-1β binds to type I IL-1 receptors. NF-κB is important in the transcription of many innate immune cell types and plays a vital role in inflammation, lymphocyte activation, cell survival, and the formation of secondary lymphoid organs (Abul K. Abbas, 2018).

Additionally, inflammasomes can be broken down into categories based on what sensor or receptor they contain. Pattern recognition receptors (PRRs) are signaling receptors of the innate immune system. PRRs recognize pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) in order to activate innate immune responses (Abul K. Abbas, 2018). The main PRRs of interest for this study are NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) and absent-in-melanoma (AIM)-like receptor (ALR), AIM2 (Mankan et al., 2012). The NLR protein sensor portion of inflammasomes, such as in NLRP3, can further be broken down into three main domains. They have an N-terminal death-fold domain (a pyrin domain for NLRP3), a central NACHT nucleotide-binding domain, and C-terminal leucine-rich repeats (LRRs). The pyrin death-fold domains interact with ASC and/or caspase 1. The NACHT domain has been implicated in the oligomerization of the proteins due to its ATPase activity while the LRRs play a role in regulation and may be involved in ligand

interaction. Rather than an NLR, absent in melanoma 2 (AIM2) inflammasomes contain a pyrin domain for ASC recruitment and a DNA-binding HIN domain (Latz et al., 2013).

When functioning properly, inflammasomes play a key role in innate immunity and can recognize activators such as microbe-associated molecular patterns (MAMPS), danger associated molecular patterns (DAMPs) from tissue damage or cell stress, and cytosolic double-stranded DNA (C. A. Yang & Chiang, 2015) (Mankan et al., 2012). However, there is growing evidence that highlights the potential roles inflammasomes play in various autoinflammatory or inflammatory disorders. The NLRP3 inflammasome has been suggested to play a role in autoinflammatory diseases, such as Schnitzler's syndrome, as well as metabolic and inflammatory conditions such as obesity, type 2 diabetes, gout, intestinal inflammation and cryopyrin-associated periodic syndromes (CAPS) (Pellegrini et al., 2017) (Menu & Vince, 2011). The AIM2 inflammasome recognizes cytosolic dsDNA, thus it has been speculated that it too may play a role in autoinflammatory conditions (Mankan et al., 2012). Both NLRP3 and AIM2 inflammasomes have been implicated in systemic lupus erythematosus (SLE) as contributing to severity of the disease (C. A. Yang et al., 2015). Additionally, overactivation of inflammasomes has been shown to lead to host death through disseminated intravascular coagulation (DIC) brought about by pyroptosis, a type of cell lysis with ruptured cell membranes. While blood clotting is normal and necessary to stop bleeding, DIC is a pathologic state with systemic activation of blood coagulation which doubles the mortality rate in patients with sepsis. It has been shown that activation of inflammasomes with lipopolysaccharide (LPS) leads to the release of tissue factor (TF) which then initiates coagulation cascades, leading to DIC when over activated (Wu et al., 2019). Further studies into the mechanisms at play in inflammasome activation and regulation could prove beneficial for a plethora of disease states.

Several post-translational modifications have been implicated in the regulation and activation of inflammasomes (J. Yang et al., 2017). Histone deacetylases (HDACs) are named for their ability to deacetylate histone proteins and therefore regulate gene expression through changing the chromatin

structure and causing repression or activation of transcription. However, they have also been shown to regulate acetylation of thousands of non-histone proteins post-translationally (Das Gupta et al., 2016). In studying the effect of HDAC inhibitors as anticancer agents, secretion of IL-1β was also found to be inhibited (Carta et al., 2006) (Leoni et al., 2002). However, pan-HDAC inhibitors, such as trichostatin A (TSA) which inhibit all HDACs rather than a select few, can cause adverse effects in healthy tissues such as inhibition of cell proliferation, which can be particularly detrimental to tissues with high cell turnover rates such as intestinal epithelia or hematopoietic cells. TSA can also inhibit apoptosis, an important physiological process in maintaining tissue homeostasis (Papeleu et al., 2005). Previous studies have indicated that HDAC inhibition causes hyperacetylation of HMGB1, a stress-response protein that also participates in the sensing of cytosolic DNA (Yanai et al., 2009) (Evankovich et al., 2010). Given that HMGB1 has also been implicated in AIM2 inflammasome function (unpublished data), we hypothesized that HDACs may regulate AIM2 inflammasome function via cytosolic DNA sensing. This study therefore has two AIMS: 1) To examine post-translational mechanisms that regulate cytosolic DNA sensing and inflammasome activation, and 2) to assess the specific HDAC enzymes involved which may pave the way for more targeted therapies.

**RESULTS**

To evaluate any association between HDAC genes and NLRP3, human embryonic kidney 293T (HEK293T) cells were transfected with expression vectors encoding HDACs 1-10 tagged with a FLAG epitope (Figure 1A) and NLRP3 with an eGFP tag (Figure 1B). After incubating the total cell lysates on a GFP-Trap affinity column, eluates were probed with antibodies against FLAG (Figure 1C) or GFP (Figure 1D). This demonstrated an interaction between eGFP-NLRP3 and HDACs 3, 4, 5, 6, and 10 (Figure 1C).

Based on other ongoing work in the lab that had implicated HDAC4 as an HMGB1-interacting protein (unpublished data, William Willis), we chose to focus our interests on HDAC4, a class IIa HDAC. To examine whether HDAC4 interacts with the inflammasome sensor AIM2, we examined whether

HDAC4 co-localizes with AIM2 via confocal microscopy. Mouse embryonic fibroblasts (MEFs) were stimulated with lipopolysaccharide (LPS) to prime the inflammasomes to begin production of pro-IL-1β. Poly dA:dT, a repetitive synthetic double-stranded DNA sequence of poly(dA-dT):poly(dT-dA), was then transfected into the cells to activate the AIM2 inflammsome. High mobility group box 1 protein (HMGB1) was also examined due to its role in cytosolic DNA sensing. HMGB1 was shown to colocalize with HDAC4 as well as AIM2, suggesting that HDAC4, HMGB1, and AIM2 form a complex together (Figure 2A). Since confocal microscope technology works by using a pinhole aperture to collect only that light which is in focus in a focal plane (i.e. an optical slice), it is cable of imaging samples at multiple points along the Z-axis (a Z-stack). Orthogonal images from a Z-stack of cells showing HMGB1, HDAC4, and AIM2 interaction were also collected in order to verify colocalization in three dimensions (Figure 2B).

HMGB1, which is normally present in the nucleus, moves to the cytosol by a lysine acetylation-dependent mechanism (Bonaldi et al., 2003). Since lysine side chains have a positive charge, and DNA has a negative charge, acetylation of HMGB1 could decrease its ability to bind DNA. We therefore hypothesized that HDAC4 regulates HMGB1 DNA binding. To test whether this was the case, we generated an HDAC4 knockout cell line in mouse embryonic fibroblasts (MEFs).

CRISPR (clusters of regularly interspaced short palindromic repeats) was used to knock out (KO) HDAC4 from MEFs. We then examined HMGB1 DNA binding in WT vs. HDAC4 KO MEFs by *in situ* pulldown assay. For this, cells were transfected with biotinylated poly dA:dT or a mock transfection containing only the lipid-transfection reagent. Cells were then lysed and incubated with streptavidin-HRP coated beads to capture the biotin-labeled probes and associated proteins. Eluates from the beads were analyzed by Western blot for HMGB1 and HMGB2, a homologous HMG protein family member. Although HMGB1 didn't bind the bead in either cell type, HMGB2 was pulled down with the biotin-dA:dT only in the WT MEFs (Figure 3A, left panel). A Ponceau stain was used to evaluate total protein levels to

demonstrate equal protein loading throughout treatment groups (Figure 3A, right panel). A similar result was found in RAW 264.7 cells, a mouse macrophage-like cell line, when HDAC4 was pharmacologically inhibited. The RAW cells were treated with a mock transfection control, poly dA:dT, or poly dA:dT and Tasquinimod, an HDAC4 inhibitor. After the cells were harvested, an *in vitro* pulldown assay was performed either with or without the addition of biotinylated poly dA:dT. For the in vitro pulldown, cytosolic extracts were probed with biotinylated poly dA:dT compared to a negative control without the biotin-labeled probe. The extracts were then incubated with streptavidin-coated beads to isolate proteins that bound to the biotinylated probe. The bound proteins were then eluted from the beads and analyzed by Western blot. Western blots of the eluates revealed that cells treated with Tasquinimod had reduced HMGB2 binding (Figure 3B).

The above results suggested that HDAC4 may regulate AIM2 inflammasome function in-part via the DNA sensing function of HMG proteins. To further test this idea in a primary cell line, we measured IL-1β secretion in response to inflammasome activation in bone-marrow derived macrophages (BMMs) by enzyme-linked immunosorbent assay (ELISA). The cells were divided into four initial treatment categories; vehicle control, LPS, LPS and ATP, or LPS and dA:dT. The LPS was used to prime the inflammasomes to begin production of pro-IL-1β while the ATP and dA:dT were used to stimulate NLRP3 or AIM2 inflammasomes, respectively. To test the effects of HDAC inhibition, the treatment groups were further broken down into four pharmacological inhibitor conditions including a vehicle control, Tasquinimod, TSA, or MS-275. Tasquinimod was used to inhibit HDAC4, TSA served as a pan-HDAC inhibitor, and MS-275 inhibited HDACs 1 and 3. No IL-1β secretion was observed in the cells that had not been primed with LPS or activated with ATP or dA:dT, and limited IL-1β secretion occurred in LPS-primed cells treated with TSA or MS-275. As expected, stimulation of LPS-primed cells with ATP or dA:dT induced high levels of IL-1β production. All HDAC inhibitors reduced inflammasome activation. While the IL-1β suppression was less with TQ, it was also the most targeted of the inhibitors (Figure 4A).

Finally, to examine whether HDAC4 is involved in pro-IL-1β processing we used a synthetic inflammasome reconstitution assay in MEFs. To reconstitute IL-1β processing in MEFs, which lack the ability to form functional inflammasomes (data not shown), we transfected pro-IL-1β, the precursor form of IL-1 β in WT and HDAC4 KO MEFs. On their own, both WT and HDAC4 KO MEFs did not process and secrete IL-1β. However, transfecting pro-IL-1β into WT cells resulted in a substantial increase in IL-1β in the cell culture media. In contrast, IL-1β levels were barely detected in the HDAC4 KO cells under the same conditions. By transfecting HDAC4 back into these knock out cells, a partial rescue affect was observed. To examine whether the apparent dependence on HDAC4 for pro-IL-1β processing was caspase-1 dependent, cells were then transfected with both pro-IL-1β and caspase-1. Notably, the large differences between WT and HDAC4 KO MEFs noted with pro-IL-1β transfection alone were not present in the cells transfected with both expression vectors; IL-1β levels in the culture media were similar in all experimental treatment groups(Figure 4B). These results suggest that HDAC4 may regulate IL-1β processing independently of caspase-1 (Figure 4C).

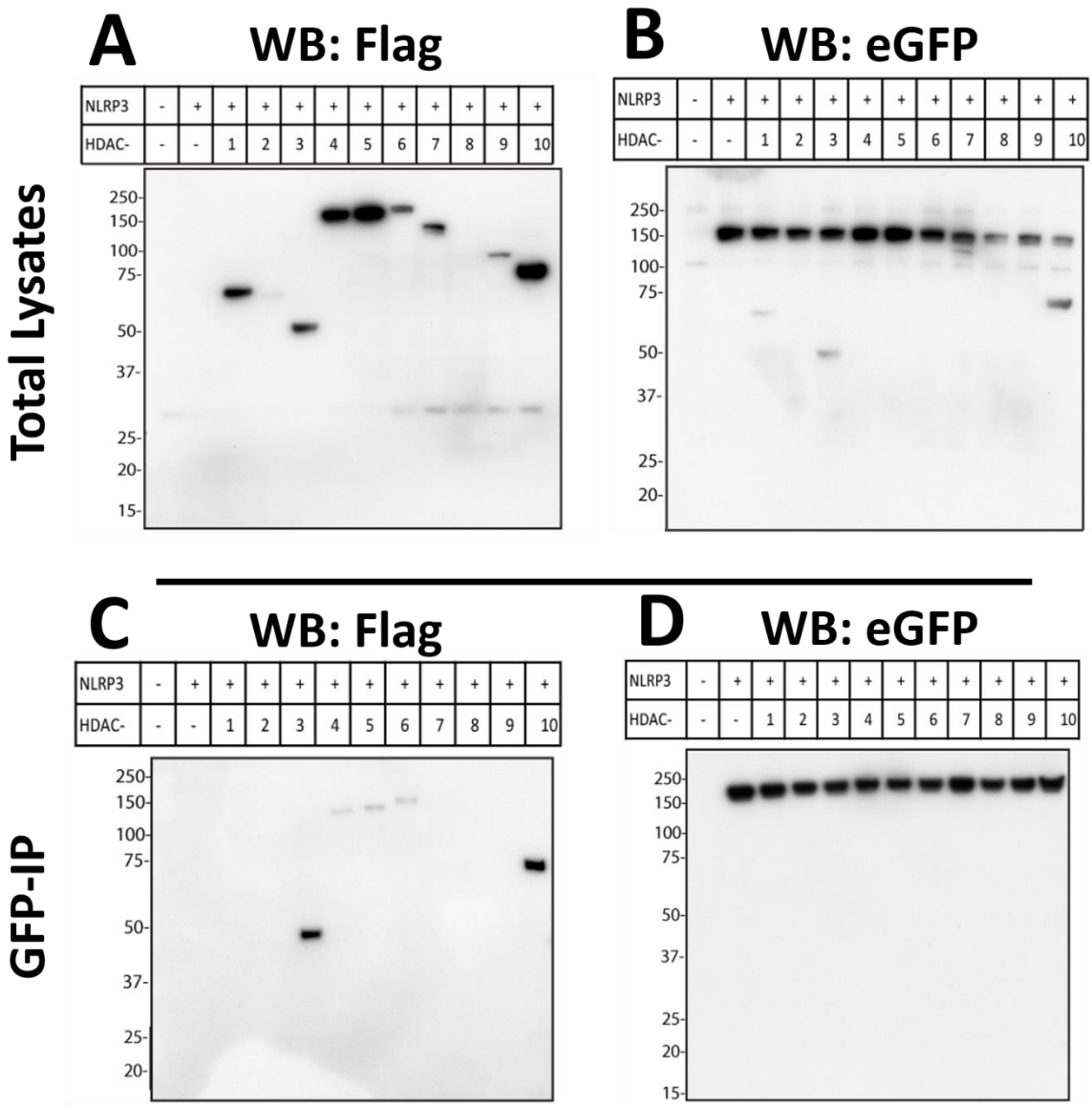


**Figure 1: HDACs associate with the NLRP3-inflammasome sensor**

Various FLAG-tagged HDACs were co-transfected with eGFP-tagged NLRP3 in HEK293T cells. A,B: Total cell lysates show expression of the exogenous HDAC proteins(A) and eGFP-NLRP3(B). C,D: Total cell lysates were incubated on a GFP-Trap affinity column. Eluates were probed with anti-Flag (C) or GFP(D), indicating that HDAC 3,4,5,6, and 10 interacted with eGFP-NLRP3.

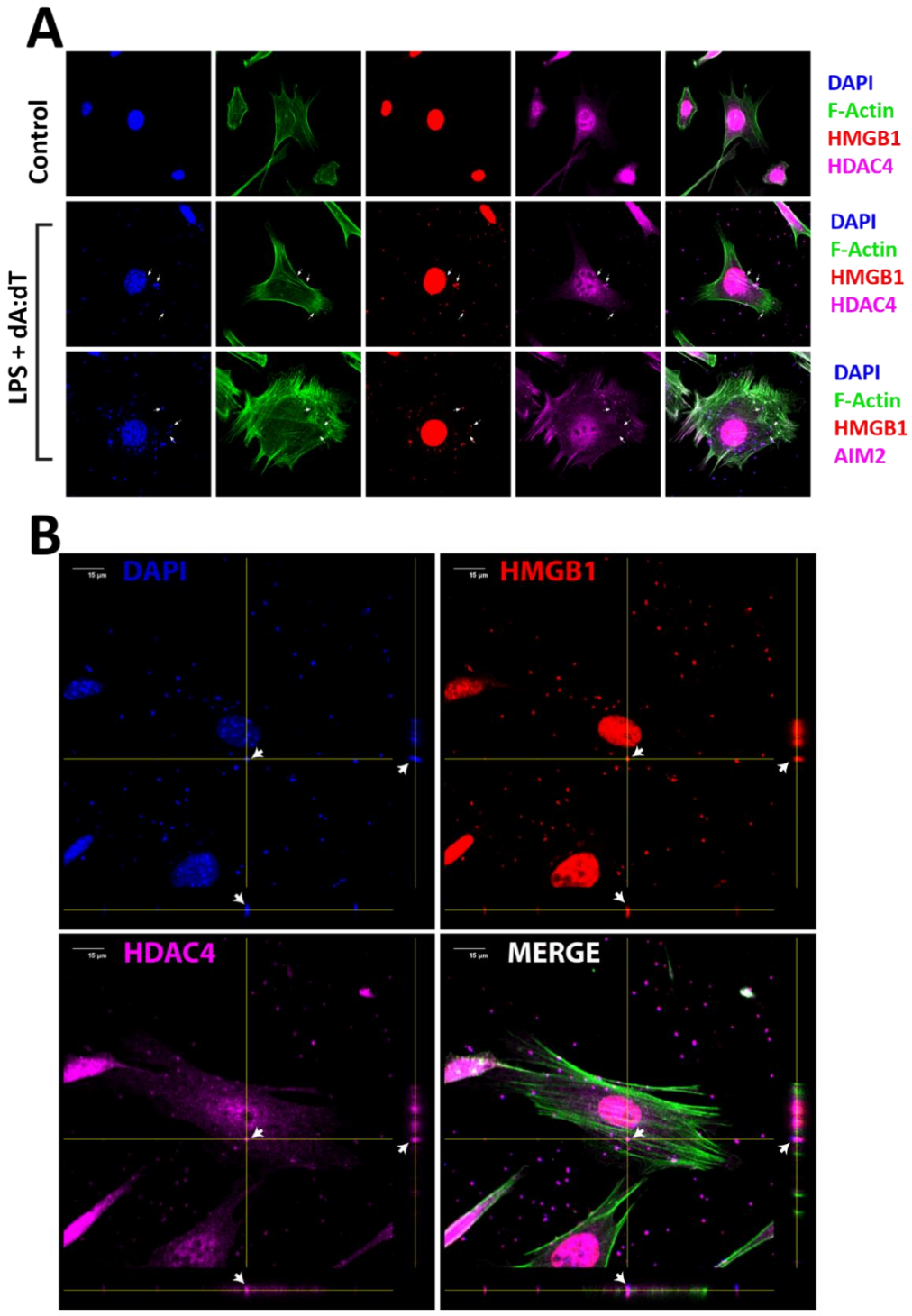


**Figure 2: HDAC4 and HMGB1 colocalize with the AIM2 inflammasome**

A: Mouse embryonic fibroblasts (MEFs) were stimulated with LPS plus poly dA:dT followed by confocal imaging. Middle panel: HMGB1 and HDAC4 show punctate signals, which localize with DAPI-stained poly dA:dT. B: Orthogonal images from a Z-stack projection of LPS + poly dA:dT treated cells confirms localization of HMGB1, HDAC4, and poly-dA:dT signals in the X-Y, X-Z, and Y-Z axes

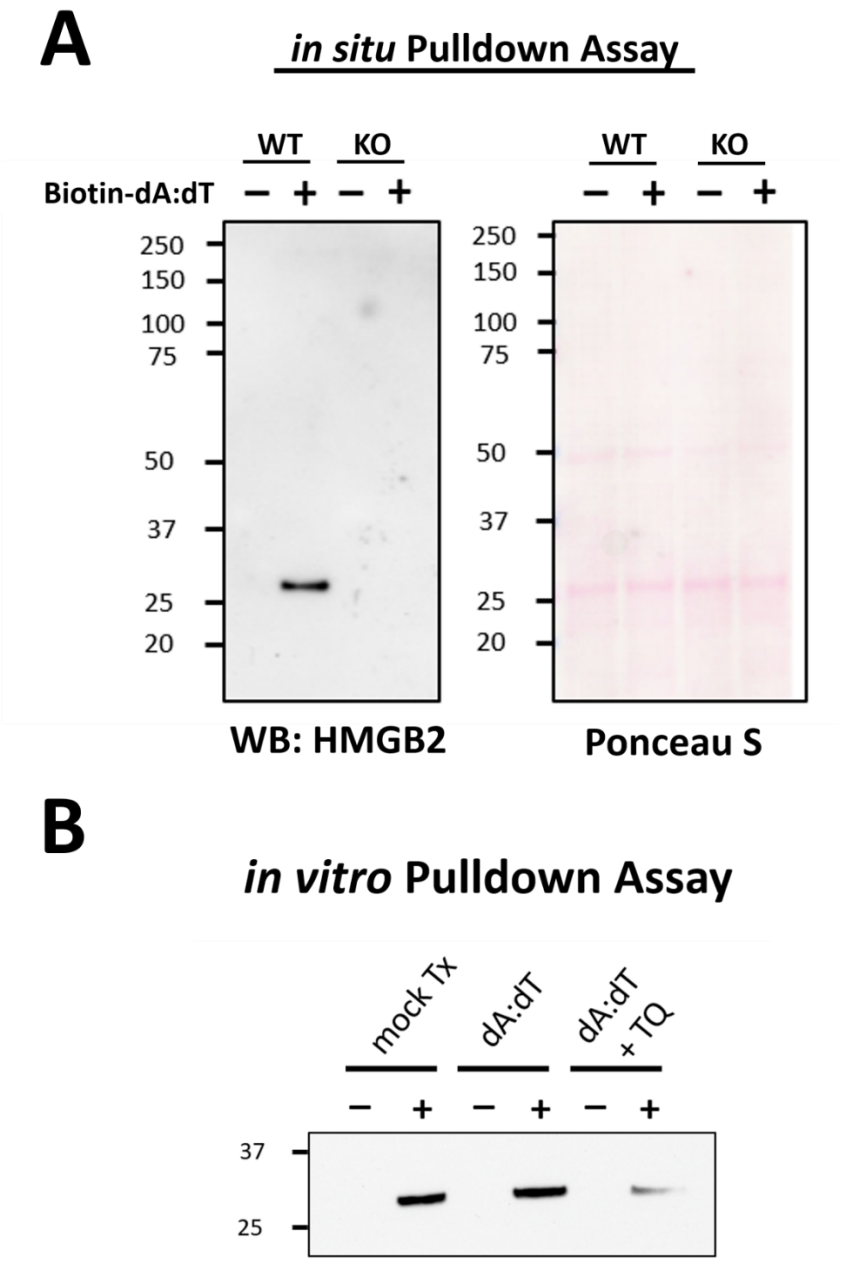


**Figure 3: HDAC4 KO or pharmacological inhibition suppresses HMGB2 DNA binding**
A: WT or HDAC4 knockout MEFs were transfected with biotinylated poly dA:dT (+), or a mock transfection with the lipid-transfection reagent only (-). No HMGB2 binding to poly dA:dT was detected in the HDAC4 knockout cells. B: RAW 246.7 cells were treated with a mock transfection, poly dA:dT, or poly dA:dT and Tasquinimod (TQ). In vitro pulldown assays were then performed using biotinylated poly dA:dT (+) as a probe, or no probe as a negative control (-). Less HMGB2 binding to poly dA:dT was detected in cells treated with TQ.

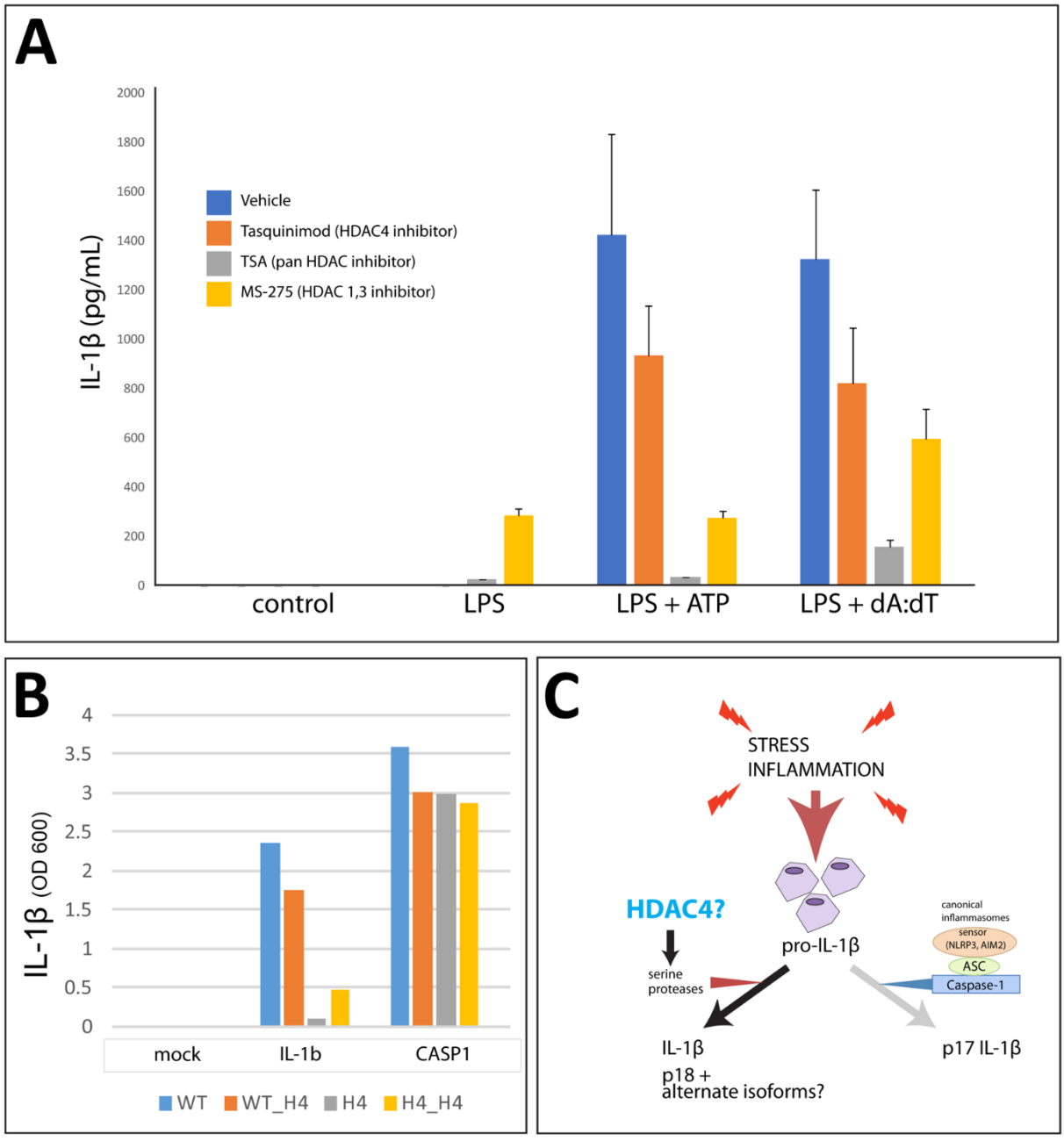


**Figure 4: HDACs regulate inflammasome activation**

A: Mouse bone-marrow derived macrophages (BMMs) were pre-treated with the HDAC inhibitors Tasquinimod (25 µM), MS-275 (25 µM), or TSA (100 nM) prior to priming with LPS (100 ng/mL overnight). Cells were then stimulated with ATP or poly dA:dT to induce respective NLRP3 or AIM2 inflammasome formation. B: WT or HDAC4 knockout MEFs were transfected with pro-IL-1β alone or with caspase-1, with or without HDAC4 transfection. HDAC4 knockout cells showed limited ability to process IL-1β, which could be partially rescued by HDAC4 transfection. WT and HDAC4 KO cells showed similar ability to process IL-1β when also transfected with caspase-1, suggesting that HDAC4 may regulate caspase-1 independent IL-1β processing, as depicted in (C).

**DISCUSSION**

Previous studies have highlighted the importance of post-translational modifications in regard to inflammasome activation and regulation. In macrophages, a phagocytic cell of the innate immune system, ASC must be phosphorylated in order for NLRP3 and AIM2 inflammasomes to be activated. However, this same ASC phosphorylation was unnecessary for successful inflammasome activation in bone marrow derived dendritic cells, another phagocyte. Even more specific are the different types of phosphorylation events facilitated by different enzymes. For example, phosphorylation of ASC as previously mentioned in macrophages from Syk and Jnk kinases led to enhanced inflammasome activation. In contrast, IκB kinase α (IKKα) functions to negatively regulate NLRP3 inflammasome activation, by reducing the translocation of ASC from the nucleus to the cytoplasm. There are also other post-translational modifications in addition to phosphorylation. Ubiquitination of NLRP3 has been shown to negatively regulate inflammasome activation while the same modification of ASC can activate inflammasomes. While most post-translational modifications are regulated by enzymes, and therefore pose possible therapeutic targets, much remains unknown about exact mechanisms (J. Yang et al., 2017).

In this study, we demonstrated that HDAC4 is associated with both NLRP3 and AIM2 inflammasomes, which suggests that this HDAC may regulate inflammasome function through deacetylation. As a class IIa HDAC, HDAC4 was previously shown to not act on acetylated histones as with other HDACs and so was thought to lack enzymatic function. However, HDAC4 was recently shown to be an active enzyme capable of deacetylating myosin heavy chain, PGC-1α, and Hsc70 in skeletal muscle demonstrating that HDAC4 can deacetylate cytosolic and non-histone nuclear proteins (L. Luo et al., 2019). While we were conducting this study, another paper was published which showed that SIRT2, a cytosolic $NAD^+$-dependent deacetylase, was necessary to regulate NLRP3 inflammasomes. More specifically SIRT2 functions as a suppressor of NLRP3 inflammasomes, which contributes to the

maintenance of hematopoietic stem cells (HSC). As HSCs age they have reduced SIRT2 expression which leads to activation of the NLRP3 inflammasome and increased mitochondrial stress (H. Luo et al., 2019).

In contrast, our data suggests that HDAC4 is a positive regulator of inflammasome function, rather than as suppressor. We demonstrated that both pharmacological inhibition and knocking out HDAC4 through gene editing reduced the secretion of IL-1β. By transfecting HDAC4 back into these cells there was a partial rescue of IL-1β secretion. The rescue effect may have only been partial due to the low transfection efficiency of the HDAC4 constructs. Indeed, the transfection efficiency for plasmid transfection in this particular MEF line is 10%, at best (unpublished observations). Alternatively, the partial rescue effect could have indicated that HDAC4 cooperates with other, yet to be identified factors. Experiments are currently underway using adenoviral transduction, which is much higher efficiency, to rescue HDAC4 expression in the knockout MEFs.

Interestingly, the HDAC4 rescue effect was lost when caspase-1 was transfected into these same cells, with no clear difference between WT and HDAC4 KO cells. This suggests that HDAC4 regulates inflammasome activity independently of caspase 1, the classic canonical pathway for inflammasome function. Further work is needed to test the hypothesis that HDAC4 regulates inflammasome signaling via caspase 1 independent IL-1β processing and to clarify the mechanisms involved. The NLRP3 inflammasome can sometimes be activated non-canonically, through caspase 11, which could be where HDAC4 is functioning (Pellegrini et al., 2017). As it has already been suggested that the affects from NLRP3 deacetylation are independent of caspase 3, exploring alternative caspases, such as caspase 11, could prove a beneficial avenue for exploration (H. Luo et al., 2019). Going forward we also hope to further explore the use of the HDAC4 inhibitor Tasquinimod in mouse models of inflammation and autoimmune disease. Preliminary studies in Luciferin mice showed a 54.33% reduction in NF-kB activity in mice treated with Tasquinimod compared to those treated with a vehicle control (Figure 5).

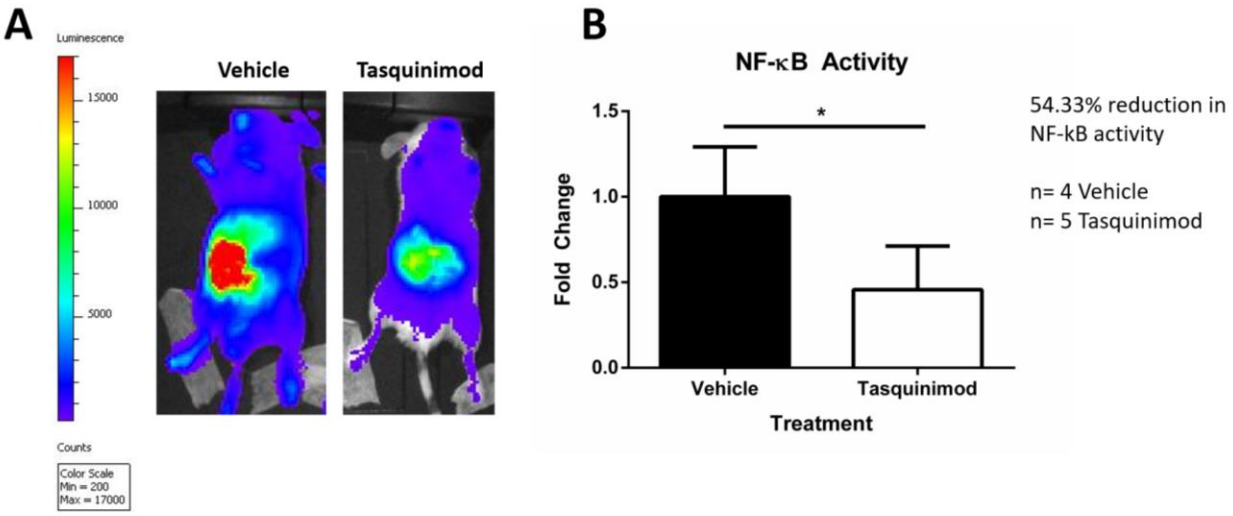


**Figure 5: Tasquinimod reduces NF-kB Activity in LPS-induced systemic inflammation**
A: NF-kB-luc reporter mice were gavaged with either vehicle or Tasquinimod (25 mg/kg) 26 hours and 2 hours before IP LPS injection (1 mg/kg). NF-kB activity determined by IVIS 4 hours later. B: Analysis by Student-Unpaired T-test *p<0.05

This work also has important potential implications for DNA cytosolic DNA sensing and AIM2 inflammasome function. High mobility group box 1 protein, or HMGB1, normally resides in the nucleus where it helps to regulate gene transcription, DNA repair, and chromatin structure. HMGB1 is capable of moving across the nuclear membrane in both directions but is kept in the nucleus through the presence of two nuclear localization signal (NLS) motifs in the HMGB1 polypeptide. However, acetylation of HMGB1 within the NLS regions causes relocation to the cytoplasm (Bonaldi et al., 2003).

HMGB proteins have been described as promiscuous sensors of nucleic acids that help to activate toll-like receptors (TLRs) 3, 4, and 9 to elicit an immune response , and HMGB1 as well as HMGB2 have been shown to play a direct role in cytosolic DNA sensing (Yanai et al., 2009). However, as noted above, HMGB1 undergoes hyperacetylation when translocating from the nucleus to the cytosol. Lysine acetylation changes the charge of the side-chain from positive to negative (Lu et al., 2014) and since DNA is negatively charged, hyperacetylation could interfere with the ability of HMGB proteins to bind to

and sense nucleic acids. This suggests that HMGB proteins may require a deacetylation event after undergoing nuclear to cytosolic translocation in order to function as an immunogenic nucleic acid sensing protein. Our data supports this idea, since we found that HDAC4 loss of function greatly impaired the ability for HMGB2 to sense and bind to DNA. Moreover, HMGB1 that was bound to poly dA:dT also colocalized with HDAC4 and AIM2 inflammasomes. This could suggest an HDAC4-dependent route for HMGB proteins to mediate immune responses through activation of inflammasome complexes. Additionally, HMGB proteins have been associated with many of the same inflammatory and autoimmune conditions that inflammasomes have been implicated in, such as systemic lupus erythematosus (Willis et al., 2018) (C. A. Yang et al., 2015).

## MATERIALS AND METHODS

### Cell lines:

RAW 264.7 cells (TIB-71), 293T cells (CRL-3216), and were purchased from ATCC. MEFs were harvested at E13.5 from a C57BL/6 mouse and immortalized with the NIH-3T3 method.

### SDS-PAGE and Western blotting

For SDS-PAGE samples were incubated in loading buffers with 100 mM DTT at 95°C for 5 min and protein samples were resolved on Bolt™ 4-12% BisTris Plus Gels (Thermo Scientific, Cat # NW04120BOX). Proteins were transferred with protein transfer buffer (48mM Tris, 390 mM glycine, 0.05% SDS, and 20% methanol) in a TE-22 mini-transfer tank (GE Biosciences) onto nitrocellulose membranes. The transfer was conducted at 450 mA for 90 min. Membranes were then stained with Ponceau S to demonstrate equal loading followed by blocking in 5% BSA/ TBS for 60 min at RT. Immunoblot using chemiluminescent HRP substrate (Thermo Scientific SuperSignal™ West Dura Extended Duration Substrate, Cat# 34075) was performed as described (Willis et al., 2013).

### GFP-Trap affinity assays

Human embryonic kidney 293T, or HEK293T, cells were transfected with various constructs expressing HDAC-FLAG fusion proteins. These cells were then co-transfected with eGFP-tagged NLRP3. Following confirmation of successful transfection, which was demonstrated through western immunoblotting with the total cell lysates, the lysates were incubated on a GFP-Trap affinity column (Chromtek GMBH). Bound proteins were eluted in SDS-loading buffer, size-fractionated by SDS-PAGE, and analyzed by Western blot with anti-FLAG and anti-GFP antibodies (Thermofisher scientific).

**Confocal microscopy**

Mouse embryonic fibroblasts (MEFs) were plated at a concentration of 2.0 x $10^4$ cells/mL in 12 well plates onto 18 mm, #1.5 poly-D-lysine coated coverslips (Neuvitro). Some cells were then stimulated with 2000ng of LPS 24 hours later and left to incubate for 1 hour at 37°C. Lipofectamine 3000 was used to transfect dA:dT into some cells which incubated for another 90 minutes at 37°C. The media was then removed, the coverslips were rinsed with phosphate buffered saline (PBS), and then fixed with 4% formaldehyde for 10 minutes at room temperature (RT). The cells were permeabilized with 0.1% Triton X-100 for 5 minutes, washed with PBS + 0.02% Tween-20 then PBS before being blocked with 1% BSA + 10% normal goat serum in PBS for 1 hour at RT. After being divided into two groups, the cells were incubated in primary antibody overnight at 4°C. Group 1 was probed for HMGB1 (mouse monoclonal [IF3], Ab190377, 1:1000) and HDAC4 (rabbit polyclonal, Ab12172, 1:250). Group 2 was probed for HMGB1 and AIM2 (rabbit polyclonal, #sc-137967, 1:50). The following day the cells were washed then incubated in secondary antibodies for 2 hours at RT, with care taken to avoid exposure to light (Alexa647, anti-rabbit IgG, 1:500 and Alexa555, anti-mouse IgG, 1:500). Finally, the cells were probed for F-actin using Alexa488 phalloidin before the coverslips were mounted in Prolong Diamond mounting medium with DAPI. After curing for 24 hours the slides were imaged using the Olympus Confocal FV-3000. The confocal images were processed on Olympus Fluoview software.

***In vitro* pulldown assay**

RAW 246.7 cells were seeded into 10 cm plates in RPMI (+10% FBS, 1% PS) at a concentration of 4.5 x $10^6$ cells/plate. The LPS groups were stimulated with 100 ng/mL of LPS 3 hours after seeding. After 24 hours the cells were treated with either a control/mock transfection or poly dA:dT 2 µg/mL transfection, using lipofectamine 3000, for 4 hours at 37°C. The cells were then removed from the incubator, placed on ice, and washed with ice-cold PBS with $Ca^{2+}$ and $Mg^{2+}$. The cells were scraped into 1mL PBS then pelleted at 2000 xg for 5 minutes at 4°C. After aspirating the PBS the cells were resuspended for fractionation in 500µL buffer A (+PIC, 0.2mM PMSF, 0.5mM DTT). The cells were left to swell on ice for 15 minutes before 31.3µL of 10% NP-40 was added, vortexed, and then immediately centrifuged at 2000 xg for 5 minutes at 4°C. The supernatant, which is the cytoplasmic extract, was transferred to a new microcentrifuge tube, and a BCA assay was used to assess the protein concentration. Streptavidin MagneSphere® Paramagnetic Particles (Promega) were used for the biotin-pulldown assay. Pulldown reactions consisted of 500 µg cytosolic extract with 10 µL of 0.1 µg/ mL biotinylated poly dA:dT. Reactions were incubated for 20 minutes at RT. The bound proteins were eluted from the beads in 50µL of 2x SDS-loading buffer then heated for 5 minutes at 95°C. Samples were then run by SDS-PAGE and probed for HMGB2 by western blot using HMGB2 antibody from Cell Signaling (Cat# 14163).

***In situ* pulldown assay**

Cells were transfected with biotinylated poly dA:dT or a mock transfection with the lipid-transfection reagent only. 4 hours after the transfection, cells were lysed in a hypotonic buffer to obtain cytosolic extracts as noted above. 500 µg Lysates were then incubated with streptavidin-coated paramagnetic beads (Promega) for 20 minutes at room temperature, washed 3x in 500 uL PBS, and eluted in 40 uL of 1x SDS-loading buffer. Samples were size-fractionated on SDS-PAGE gels and analyzed for HMGB2 binding by Western blot.

**ELISA and plasmid transfections**

To measure IL-1β secretion in cell culture media, 50 µL of cell culture media was measured via a commercially available mouse IL-1 β ELISA kit (Invitrogen, Cat# BMS2002) per the manufacturer's instructions. For each experimental group, 3 experimental replicates were analyzed and loaded as duplicates on the ELISA plate. Plasmid transfections were conducted with Lipofectamine 3000 (Invitrogen) per the manufacturer's protocol.

**HDAC4 knockout with CRISPR**

Immortalized MEFs were co- transfected with pCas-Guide (Origene) encoding the HDAC4 targeting guide RNA TCCCGCAATGCAGGTTCCAA (Figure 6) and the linearized donor selection cassette LoxP-EF1A-tGFP-P2A-Puro-LoxP (Origene Cat# KN507619D). The knockout strategy occurs via a double-stranded break mediated non-homologous end-joining (NHEJ) mechanism, where incorporation of the donor selection cassette in one allele at the site of repair causes a high probability of reading frame shift-inducing insertion/ deletion (Indel) events on the opposing allele. After transfection, the cells were passaged 6 times to ensure that all selection cassette DNA had been stably integrated into genomic DNA (i.e. to dilute out any selection cassette DNA that may have maintained an episomal presence during earlier passages). Growth media was then supplemented with puromycin (4 µg/ ml) to select for drug-resistant clones. The polyclonal pool of HDAC4 gene-disrupted, GFP positive cells were then seeded out into 96-well plates for single-cell cloning by limiting dilution. 40 single-cell derived clones were screened for HDAC4 knockout by western blot, resulting in several clones with bi-allelic HDAC4 knockout. Loss of HDAC4 expression and double-stand break targeting was verified Western blot, and PCR of the genomic DNA, respectively.

**In Vivo Imaging System (IVIS)**

BALB/C-Tg(NFκB-RE-luc)-Xen mice were purchased from Caliper Life Science (Hopkinton, MA). All animals were housed at The Ohio State University Wexner Medical Center (OSUWMC) in a BSL-3 barrier facility; maintenance and protocols were specifically approved for this study by the Institutional

Animal Care and Use Committee through The University Laboratory Animal Resources at OSUWMC. BALB/C-Tg(NFκB-RE-luc)-Xen mice were imaged as described previously. NF-kB-luc reporter mice were gavaged with either vehicle or Tasquinimod (25 mg/kg) 26 hours and 2 hours before IP LPS injection (1 mg/kg). NF-kB activity was determined by IVIS 4 hours later. Briefly, mice were given 150 mg/kg luciferin [(Gold Biotechnology, Inc.); 15 mg/mL in PBS (pH 7; unadjusted)] through intraperitoneal injection and bioluminescent signals were captured following indicated time periods using IVIS Lumina II (Xenogen). Data were quantitatively analyzed using IVIS Living Image software (v4.5; PerkinElmer).

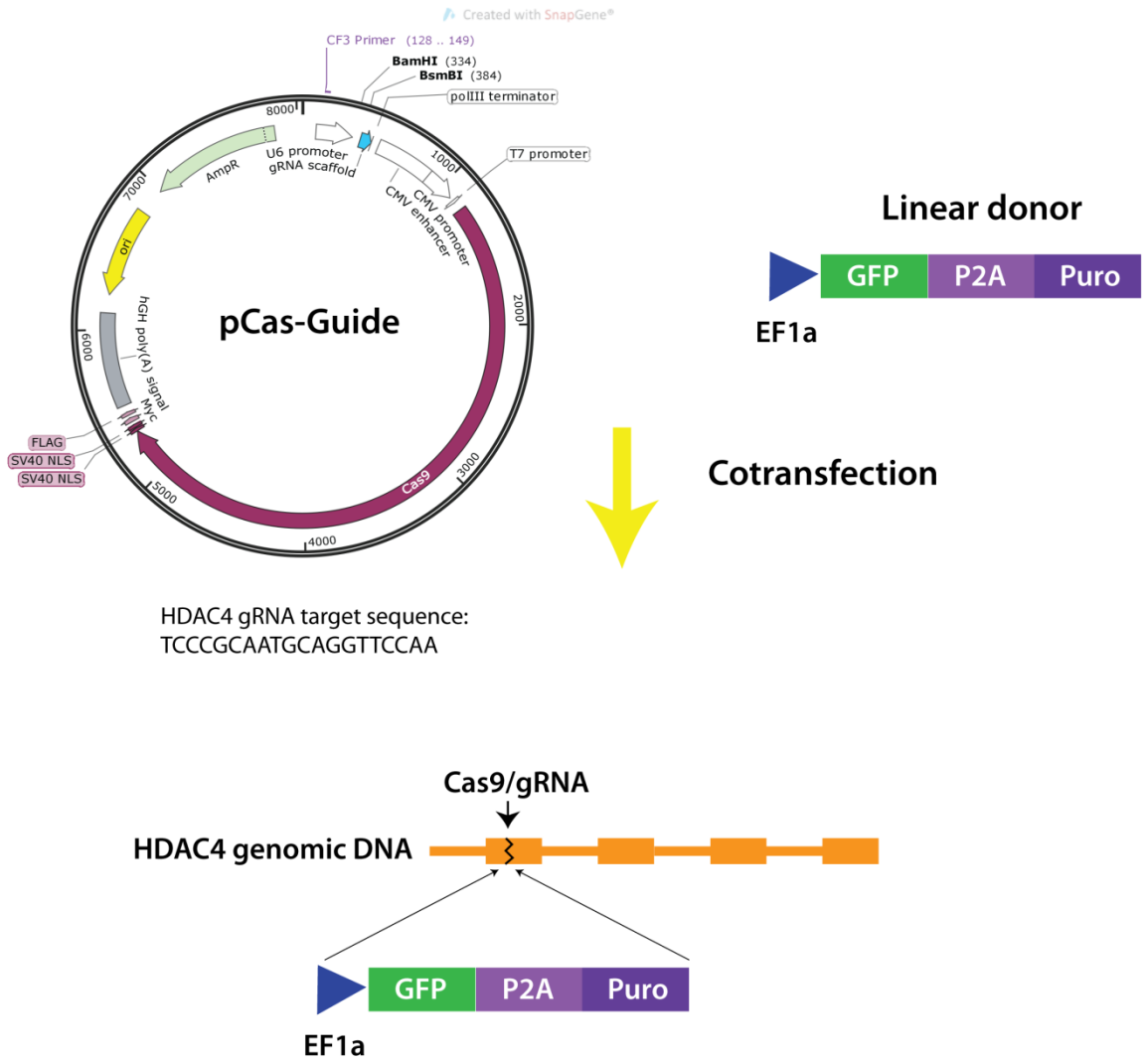


**Figure 6: HDAC4 CRISPR knockout strategy**

pCas-Guide encoding an HDAC4 targeting gRNA was co-transfected with linear donor DNA encoding GFP and puromycin resistance into MEF cells to induce gRNA-targeted double-stranded breaks in the open-reading frame of HDAC4, disrupting protein expression.

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